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# Effects of Dietary Folate and Alcohol Intake on Promoter Methylation in Sporadic Colorectal Cancer: The Netherlands Cohort Study on Diet and Cancer<sup>1</sup>

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## ABSTRACT

Sporadic colorectal cancer (CRC) is characterized by genetic and epigenetic changes such as regional DNA hypermethylation and global DNA hypomethylation. Epidemiological and animal studies suggest that aberrant DNA methylation is associated with low dietary folate intake, which is aggravated by high alcohol intake. The relationship between promoter methylation of genes involved in CRC carcinogenesis and folate and alcohol intake was investigated. Methylation of the *APC-1A*, *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>*, *hMLH1*, *O<sup>6</sup>-MGMT*, and *RASSF1A* promoters was studied using methylation-specific PCR in 122 sporadic CRCs, derived from patients with folate and alcohol intake at either the lower or the higher quintiles of the distribution. Overall, promoter hypermethylation frequencies observed were: 39% for *APC*; 33% for *p14<sup>ARF</sup>*; 31% for *p16<sup>INK4A</sup>*; 29% for *hMLH1*; 41% for *O<sup>6</sup>-MGMT*; and 20% for *RASSF1A*. For each of the tested genes, the prevalence of promoter hypermethylation was higher in CRCs derived from patients with low folate/high alcohol intake ( $n = 61$ ) when compared with CRCs from patients with high folate/low alcohol intake ( $n = 61$ ), but the differences were not statistically significant. The number of CRCs with at least one gene methylated was higher (84%) in the low folate intake/high alcohol intake group when compared with the high folate intake/low alcohol intake group (70%;  $P = 0.085$ ). Despite the size limitations of this study, these data suggest that folate and alcohol intake may be associated with changes in promoter hypermethylation in CRC.

## INTRODUCTION

In addition to hereditary components, known risk factors for CRC<sup>3</sup> are related to lifestyle and environment such as smoking and a Western diet (high meat, energy, and alcohol intake and low fruit, vegetable, and fiber intake; Ref. 1). Although the majority of epidemiological studies point to fruit and vegetable intake as protective for CRC (2–11), the molecular mechanisms for this protection remain to be clarified. It is hypothesized that folate, one of the vitamins mainly found in green leafy vegetables, is in part responsible for the inverse association with CRC risk. The effects of low folate intake are aggravated by high alcohol intake (7), probably because of degradation of folate in the colon by acetaldehyde, the first metabolite of alcohol (12).

Folate, as 5-methyltetrahydrofolate, has a central role in methyl metabolism. It supplies a methyl group to convert homocysteine to

methionine, which is then converted to S-adenosylmethionine, the universal methyl donor for methylation of a wide variety of biological substrates such as DNA, RNA, and proteins. Folate deficiency is reported to be associated with the occurrence of point mutations in *K-ras* in colorectal adenomas (13) and carcinomas (14), single and double strand DNA breaks, chromosome breakage (15, 16), and global DNA hypomethylation (17).

Although the overall level of genomic methylation is actually reduced in certain tumor types, including CRCs (18, 19), hypermethylation at several gene promoters has also been reported. It has been hypothesized that global hypomethylation might induce regional *de novo* hypermethylation (20). On the other hand, a recent study by Bariol *et al.* (21) suggests that there is no relationship between global demethylation and regional hypermethylation in CRC.

To investigate whether altered DNA methylation is associated with folate and alcohol intake, we examined promoter methylation of genes that have been reported to be involved and methylated in CRC carcinogenesis, *i.e.*, *APC-1A*, *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>*, *hMLH1*, *O<sup>6</sup>-MGMT*, and *RASSF1A* (22, 23). This was done using an optimized MSP method. This study was performed with 122 CRC archival specimens derived from incident cases who participate in the prospective NLCS (24). Patients with low folate intake in addition to high alcohol intake at baseline ( $n = 61$ ) were compared with patients with high folate intake in addition to low alcohol intake ( $n = 61$ ) with respect to the methylation status of a series of gene promoters.

## MATERIALS AND METHODS

**Study Population and Sample Procurement.** The paraffin-embedded CRC samples used in this study were derived from patients who participate in NLCS. This study started in September 1986 and included 58,279 men and 62,573 women (55–69 years at baseline). At baseline, the cohort members completed a mailed, self-administered food frequency questionnaire on dietary habits and other risk factors for cancer. The study design has been described in detail elsewhere (24). Follow-up for incident cancer is established annually by computerized record linkage with all cancer registries in the Netherlands and with PALGA, a nationwide pathology database (25). Since 1989, the coverage of PALGA is 100% (26). Record linkage covering the period from 1989 up to the end of 1993 (7.3 years follow-up, with exclusion of the first 2.3 years of follow-up) resulted in 819 eligible incident, histologically confirmed CRC patients. After approval by the Medical Ethical Committee of the Maastricht University and PALGA, tissue samples were collected from 54 pathology laboratories throughout the Netherlands. Tumor tissue sample collection was started in August 1999 and was completed in December 2001.

For this pilot study, 61 CRCs of patients with high methyl donor intake [high folate intake ( $\geq 215 \mu\text{g/day}$ ) in addition to extremely low alcohol intake (0–4 g/day)] and 61 CRCs of patients with low methyl donor intake [folate intake ( $< 215 \mu\text{g/day}$ ) in addition to high alcohol intake ( $\geq 5 \text{ g/day}$ )] were selected (for selection criteria and definitions; Table 1). Among the selected patients were 68 males and 54 females (58–76 years of age at time of diagnosis), 30 Dukes A, 39 Dukes B, 31 Dukes C, and 15 Dukes D CRCs. Dukes stage was not reported in 7 cases. The distribution of the location of the tumors was colon ( $n = 84$ ), rectosigmoid ( $n = 14$ ), and rectum ( $n = 24$ ). The

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<sup>3</sup> The abbreviations used are: CRC, colorectal cancer; APC, adenomatous polyposis coli; hMLH1, human mut-L homologue; *O<sup>6</sup>-MGMT*, *O<sup>6</sup>-methylguanine DNA methyl transferase*; NLCS, Netherlands Cohort Study on Diet and Cancer; MSP, methylation-specific PCR; OR, odds ratio; CI, confidence interval; SNP, single nucleotide polymorphism; PALGA, Pathologisch Anatomisch Landelijk Geautomatiseerd Archief.

Table 1 Selection of CRC patients based on extreme intakes of folate and alcohol intake (quintile distribution)

Methyl donor group (dichotome)	Quintile combination <sup>a</sup>	Methyl donor category	Folate intake (μg/day)	Alcohol intake (g/day)	n
Low folate/high alcohol (n = 61)	F1/A5	1	0–157	≥30	7
	F1/A4	2	0–157	15–29	14
	F1/A3	3	0–157	5–14	15
	F2/A5	4	158–186	≥30	16
	F2/A4	5	158–186	15–29	6
	F3/A5	6	187–215	≥30	3
High folate/low alcohol group (n = 61)	F4/A1	7	215–255	0	19
	F5/A2	8	≥255	0.1–4	24
	F5/A1	9	≥255	0	18

<sup>a</sup> Quintiles of folate (F) and alcohol (A) intake; i.e., F1/A5 is first quintile of folate and fifth quintile of alcohol.

122 patients in this study were representative for the complete group of eligible CRC patients with respect to age, gender, Dukes stage, and tumor location distribution.

After identification by a pathologist (A. P. d. B.), genomic DNA of tumor cells was microdissected and extracted using proteinase K (Qiagen) and the Puregene DNA Isolation Kit (Gentra Systems) and stored at 4°C. Part of this set of samples has previously been characterized for *RASSF1A* promoter methylation (23).

**Food Frequency Questionnaire.** At the start of the NLCS in 1986, all participants completed a food frequency questionnaire on daily food consumption and potential confounders (e.g., smoking, occupation, physical activity, family history of cancer, drug use). The dietary section of the questionnaire, a 150-item semiquantitative food frequency questionnaire, concentrated on habitual consumption of food and beverages during the year preceding the start of the study. The questionnaire was validated against a 9-day dietary record. Daily mean nutrient intakes are calculated using the computerized Dutch food composition table. Folate intake was calculated from newly established liquid chromatography data for foods (27).

Questionnaire data of all cases and the subcohort are key-entered twice and processed in a manner blinded with respect to case/subcohort status to minimize observer bias in coding and interpreting the data. Both procedures make use of a highly standardized protocol. Subjects with incomplete or inconsistent dietary data are excluded from data analysis, according to criteria described in detail elsewhere (28). Patients with low folate intake in addition to high alcohol intake were defined as the low methyl donor group, whereas patients with high folate intake in addition to low alcohol intake were defined as the high methyl donor group (Table 1). Dietary factors adjusted for in data analyses are chosen

on the basis of a previous analysis on the association between folate and CRC (2).

**Promoter Methylation Analysis.** DNA methylation in the CpG islands of the *APC-1A*, *p14<sup>ARF</sup>*, *p16<sup>INK4a</sup>*, *hMLH1*, *O<sup>6</sup>-MGMT*, and *RASSF1A* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent MSP as described in detail elsewhere (29). In brief, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in H<sub>2</sub>O.

To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction.

All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles [normal lymphocyte DNA treated *in vitro* with *SssI* methyltransferase (New England Biolabs)], and a control without DNA. Primer sequences and PCR conditions are described in Table 2. Ten μl of each MSP reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. To study the reproducibility of the nested MSP approach, duplicate CRC specimens were analyzed. Reproducibility was 95%.

**Data Analysis.** Frequencies of promoter methylation of specific genes and frequencies and means of other variables in patients with high and low methyl donor intake were computed. Differences in frequencies of variables were tested using the  $\chi^2$  test, and differences in mean levels of variables were tested using the *t* test. Logistic regression analyses were performed to test whether there was a difference in methyl donor intake (low *versus* high) between CRCs with at least one gene methylated *versus* no genes methylated while adjusting for potential confounders. Because nine categories of methyl-donor intake were distinguished (categories 1 through 9 in Table 1), although only based on extreme levels of intake, logistic regression analyses were also performed including this variable instead of the dichotomous intake variable in the model. A *P* of 0.05 was considered to be statistically significant. All tests of statistical significance were two-sided. Data analysis was done using SPSS software (version 9.0).

## RESULTS

To enable MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tumor tissue, nested MSP was performed. Nested

Table 2 MSP primer sequences and PCR conditions

Primer set	Sense primer	Antisense primer	Amp Size (bp)	Annealing temperature (°C)	No. of PCR cycles
<i>APC-1A</i> flank	5'-TGGGYGGGGTTTGTGTTTATT-3'	5'-TACRCCACACCCAACCAATC-3'	136	56	35
<i>APC-1A</i> U <sup>a</sup>	5'-GTGTTTATTGTGGAGTGTGGTT-3'	5'-CCAATCAACAACTCCCAACAA-3'	108	60	25
<i>APC-1A</i> M	5'-TATTGCGGATGTCGGGTC-3'	5'-TCGACGAACCTCCGACGA-3'	98	60	25
<i>p14<sup>ARF</sup></i> flank	5'-TTTAGTTTGTAGTTAAGGGGGTAGGAG-3'	5'-CRCTACCCACTCCCCRTAAACC-3'	207	56	35
<i>p14<sup>ARF</sup></i> flank short	5'-GYGTTGTTTATTTTGGTGTAAAGG-3'	5'-AAATATAAACCAACRAAACCCCTCACT-3'	152	56	35
<i>p14<sup>ARF</sup></i> U	5'-TTTTTGGTGTAAAGGGTGGTGTAGT-3'	5'-CACAAAAACCCCTCACTACAACAA-3'	132	60	25
<i>p14<sup>ARF</sup></i> M	5'-GTGTTAAAGGGCGCGTAGC-3'	5'-AAAACCCCTCACTCGCGACGA-3'	122	60	25
<i>p16<sup>INK4a</sup></i> flank	5'-AGAAAGAGGAGGGGTGGTTGG-3'	5'-ACRCCRCACCTCTCTACC-3'	193	56	35
<i>p16<sup>INK4a</sup></i> flank short	5'-GGGTTGGTTGGTTATTAGAGGGT-3'	5'-RACCRTAACCAACCAATCAACC-3'	148	56	35
<i>p16<sup>INK4a</sup></i> U	5'-TTATTAGAGGGTGGGGTGGATTGT-3'	5'-CAACCCCAACCAACCAACCATAA-3'	151	60	30
<i>p16<sup>INK4a</sup></i> U short	5'-GTTGGTTATTAGAGGGTGGGGTGGATTGT-3'	5'-AACCAAACTCCATACTACTCCCCACCA-3'	124	62	25
<i>p16<sup>INK4a</sup></i> M	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	5'-GACCCGAACCGGACCGTAA-3'	150	60	30
<i>p16<sup>INK4a</sup></i> M short	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	5'-GAAACTCCATACTACTCCCCGCG-3'	115	62	25
<i>O<sup>6</sup>-MGMT</i> flank	5'-GYGTTTGGATATGTTGGGATAGTT-3'	5'-AAACTCCCACTCTTCCRAAAAC-3'	135	56	35
<i>O<sup>6</sup>-MGMT</i> U	5'-TTTTGAYGTAGAGTTTGTAGGTTTGT-3'	5'-AACTCCCACTCTTCCAAAAACAA-3'	93	60	30
<i>O<sup>6</sup>-MGMT</i> M	5'-TTTCGACGTTCTAGGTTTTCGC-3'	5'-GCATCTCTCGAAAACGAAACG-3'	81	60	30
<i>hMLH1</i> flank	5'-GGAGTGAAGGAGTTAYGGGTAAGT-3'	5'-AAAAACRATAAAACCCCTATACCTAATCTATC-3'	182	56	35
<i>hMLH1</i> flank short	5'-TTTTGATGTAGATGTTTATTAGGGTGT-3'	5'-AAAACRATAAAACCCCTATACCTAATCTATC-3'	154	56	35
<i>hMLH1</i> U	5'-TGTGTGTTTGTGTTTGTATATATTGTTT-3'	5'-ACCACCTCATCATACTACCCACA-3'	124	60	35
<i>hMLH1</i> U short	5'-TGTGTGTTTGTGTTTGTATATATTGTTT-3'	5'-ACCACCTCATCATACTACCCACA-3'	98	60	35
<i>hMLH1</i> M	5'-ACGTAGACGTTTATTAGGGTCGC-3'	5'-CCTCATCGTAACCTCCGCG-3'	115	60	35
<i>hMLH1</i> M short	5'-GTTCTGCTGTTATATATCGTTC-3'	5'-CCTCATCGTAACCTCCGCG-3'	89	60	35
<i>RASSF1A</i> flank	5'-GTTTGTGTTGATTTGGGGGAG-3'	5'-CCCRCACCTCAATAAATCAAACTC-3'	144	56	35
<i>RASSF1A</i> U	5'-GGGGTTTGTGTTTGTGTTTGT-3'	5'-AACATAACCAATTAACCCATACTTCA-3'	81	60	30
<i>RASSF1A</i> M	5'-GGGTCGTTTGTGTTTGTGTTT-3'	5'-TAACCCGATTAAACCCGTAATTCG-3'	76	60	30

<sup>a</sup> U, unmethylated DNA specific primers; M, methylated DNA specific primers.

Table 3 Prevalence of promoter methylation, clinicopathological parameters and other patient characteristics for the total number of CRCs and for the high and low methyl donor intake groups

	Total (n = 122)	Methyl donor intake		P
		High (n = 61)	Low (n = 61)	
Methylation				
Promoter methylation				
APC	47/122 (39%)	20/61 (33%)	27/61 (44%)	0.193
p14 <sup>ARF</sup>	40/120 (33%)	17/60 (28%)	23/60 (38%)	0.245
p16 <sup>INK4a</sup>	37/119 (31%)	18/59 (31%)	19/60 (32%)	0.891
hMLH1	35/122 (29%)	17/61 (28%)	18/61 (30%)	0.841
O <sup>6</sup> -MGMT	50/121 (41%)	22/61 (36%)	28/60 (47%)	0.236
RASSF1A	24/122 (20%)	9/61 (15%)	15/61 (25%)	0.172
Number of genes methylated				
0	28/122 (23%)	18/61 (30%)	10/61 (16%)	
1	25/122 (20%)	12/61 (20%)	13/61 (21%)	
2	29/122 (24%)	13/61 (21%)	16/61 (26%)	
3	18/122 (15%)	8/61 (13%)	10/61 (16%)	
4	16/122 (13%)	9/61 (15%)	7/61 (12%)	
5	4/122 (3%)	1/61 (2%)	3/61 (5%)	
6	2/122 (2%)	0/61 (0%)	2/61 (3%)	0.411
At least 1 gene methylated	94/122 (77%)	43/61 (70%)	51/61 (84%)	0.085
Clinicopathological parameters				
Dukes stage <sup>a</sup>				
A	30 (26%)	17 (29%)	13 (23%)	
B	39 (34%)	18 (30%)	21 (37%)	
C	31 (27%)	13 (22%)	18 (32%)	
D	15 (13%)	11 (19%)	4 (7%)	0.19
Location of tumor				
Colon	84 (69%)	45 (74%)	39 (64%)	
Rectosigmoid	14 (11%)	4 (7%)	10 (16%)	
Rectum	24 (20%)	12 (20%)	12 (20%)	0.223
Proximal location (yes) <sup>b</sup>	75 (62%)	36 (60%)	39 (64%)	0.498
Other patient characteristics				
Age at diagnosis (yr)	67.4	66.7	68.1	0.086
Gender (male)	68 (56%)	27 (44%)	41 (67%)	0.011
Family history of CRC (yes)	19 (16%)	9 (15%)	10 (16%)	0.803
Dietary factors				
Energy intake (kcal)	1951	2105	1797	0.002
Fiber intake (g)	27	32.9	21.2	0.019
Vitamin C intake (mg)	107	135.1	79	0.001
Iron intake (mg)	12.7	14.6	10.8	0.022

<sup>a</sup> For 7 cases, Dukes stage is not reported.<sup>b</sup> For 1 case, proximal/distal location was not determined.

MSP analysis could easily be performed for *APC-1A*, *O<sup>6</sup>-MGMT*, and *RASSF1A*, genes for which the designed MSP amplicons are relatively small, *i.e.*, <165 bp (see Table 2). However, using the flanking MSP primers originally designed for *p14<sup>ARF</sup>*, *p16<sup>INK4a</sup>*, and *hMLH1* (30), the success rate for amplification decreased with increasing size of the MSP amplicons. Bisulfite-treated DNA could not be amplified for *p14<sup>ARF</sup>* (207 bp) in 18% (22 of 121), for *p16<sup>INK4a</sup>* (193 bp) in 12% (14 of 120), and for *hMLH1* (182 bp) in 16% (20 of 123) of CRC cases. To increase the MSP success rate, MSP primers for these promoter regions were redesigned to obtain shorter amplicons (<155 bp). Using these primer sets, a subgroup of CRC cases (*n* = 52), which did and which did not amplify using the original MSP primer sets, were amplified using the redesigned MSP primers. The MSP success rate increased to 94% (49 of 52) for *p14<sup>ARF</sup>*, 90% (47 of 52) for *p16<sup>INK4a</sup>*, and 100% (52 of 52) for *hMLH1*. The concordance between the original and the redesigned short primer sets was tested and was found to be 94% (31 of 33) for *p16<sup>INK4a</sup>*, 94% (30 of 32) for *hMLH1*, and 93% (25 of 27) for *p14<sup>ARF</sup>*. Because of the rate of concordance between the different primer sets, data using both sets of primers were pooled. Genes that showed methylation using only one of both primer sets were considered as methylated.

Overall, promoter hypermethylation frequencies observed were: 39% (47 of 122) for *APC-1A*; 33% (40 of 120) for *p14<sup>ARF</sup>*; 31% (37 of 119) for *p16<sup>INK4a</sup>*; 29% (35 of 122) for *hMLH1*; 41% (50 of 121) for *O<sup>6</sup>-MGMT*; and 20% (24 of 122) for *RASSF1A* (Table 3). Representative examples of the MSP reactions are shown in Fig. 1.

For all genes, the prevalence of promoter hypermethylation was higher in CRCs derived from patients with low methyl donor intake when

compared with CRCs from patients with high methyl donor intake, however, none of these individual differences reached statistical significance. The percentage of CRCs with at least one gene methylated *versus* no gene methylated was higher (84%) in the low methyl donor intake group when compared with the adequate/high methyl donor intake group (70%; *P* = 0.085; Table 3).

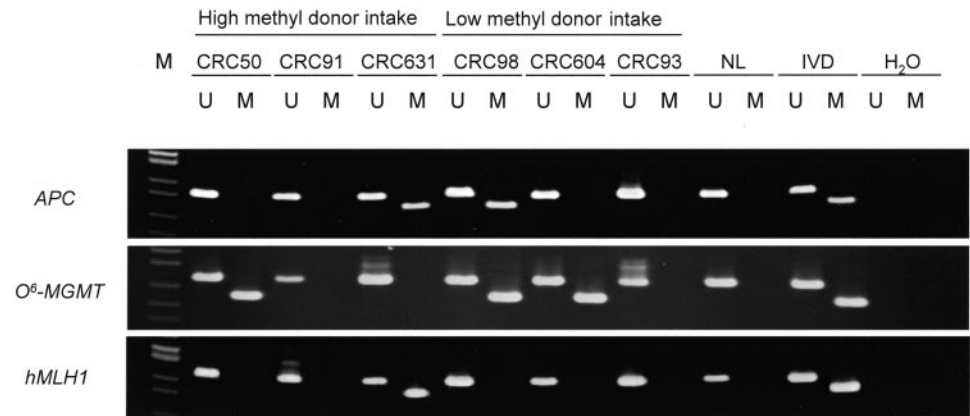
Dukes stage and location of the tumor did not differ between the two groups of patients (Table 3). Age at diagnosis was higher in patients with low methyl donor intake. There were significantly more males in the low methyl donor intake group (67%) when compared with the high methyl donor intake group (44%; *P* = 0.011; Table 3). Factors previously shown to be associated with folate intake, *i.e.*, energy-, fiber-, vitamin C-, and iron intake (2), were all significantly lower in patients with low methyl donor intake compared with patients with adequate/high methyl donor intake (Table 3).

Table 4 summarizes the Dukes stage, location of the tumor, age at diagnosis and energy-, fiber-, vitamin C-, and iron intake, none of which are associated with promoter methylation. Family history of cancer frequency was higher (25%) in patients who had none of the six studied genes methylated, compared with patients with at least one gene methylated (13%), but the difference did not reach statistical significance (Table 4). Table 4 also shows that gender is significantly associated with promoter methylation (*P* = 0.046). Sixty-one percent of patients were male in the group with at least one gene methylated *versus* 39% in the group with no genes methylated (Table 4). Caution is warranted in interpretation because these results are based on a selection of extreme intakes.

The OR for promoter methylation of at least one gene methylated



Fig. 1. Representative examples of *APC-1A*, *O<sup>6</sup>-MGMT*, and *hMLH1*-nested MSP reactions of six primary CRCs and controls. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated alleles; the presence of product in those lanes marked M indicates the presence of methylated alleles. All CRCs include amplification with the U primer set, probably a result of the presence of normal, contaminating tissue. Normal lymphocytes (NL) and *in vitro* methylated DNA (IVD) were used as negative and positive controls for *APC-1A*, *O<sup>6</sup>-MGMT*, and *hMLH1* promoter methylation, respectively. The H<sub>2</sub>O control was included in the flanking PCR and subsequently in the MSP reaction.



was 2.13 (95% CI, 0.89–5.11) for patients with low methyl donor intake *versus* high methyl donor intake. Adjustment for age, sex, energy-, fiber-, vitamin C-, and iron intake and family history of cancer revealed an OR of 1.82 (95% CI, 0.49–6.78). When the methyl donor intake variable with nine categories was modeled in a similar way, a positive association with promoter methylation of at least one gene was still observed, although not statistically significant (unadjusted OR, 1.14,  $P = 0.124$ ; adjusted OR, 1.12,  $P = 0.420$ ).

## DISCUSSION

A better understanding of the causality of CRC can be established by combining epidemiology and research on molecular mechanisms. Here, this approach was used to study whether dietary intake of folate and alcohol is associated with hypermethylation of tumor suppressor- and DNA repair genes in CRC specimens derived from the NLCS. Nested MSP analysis was optimized, and as a result, almost all CRC specimens, irrespective of the quality of the DNA, could be analyzed for hypermethylation in the promoter regions of *APC-1A*, *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>*, *hMLH1*, *O<sup>6</sup>-MGMT*, and *RASSF1A*. This optimized nested MSP approach enables high throughput promoter methylation analysis in archival, formalin-fixed and paraffin-embedded tissues for molecular epidemiology studies.

For *p14<sup>ARF</sup>*, *p16<sup>INK4A</sup>*, and *O<sup>6</sup>-MGMT*, the overall prevalence of promoter methylation, *i.e.*, 33, 31, and 41%, respectively, are in the range of the prevalence reported for CRC (22, 31, 32). For *RASSF1A*, the prevalence of promoter methylation was similar to the frequency

reported on a larger series of CRCs from the same cohort (23). For *APC-1A* and *hMLH1*, the overall prevalence is higher than reported thus far (30, 33). This difference might be because the CRC material used in this study is obtained from patients from a different geographic area (the Netherlands) compared with the patient material used in the other studies (United States).

Although not significant, our results indicate that methyl donor deficiency is associated with overall methylation of multiple genes, an effect which is stronger than the effect on specific promoter regions itself. The association, although not statistically significant, between the nine different categories of methyl donor intake and promoter methylation of at least one gene supports this. However, these results have to be interpreted with caution because only extreme categories were used, and intermediate categories of methyl donor intake in this pilot study were omitted. In addition, stratification of patients was done primarily on folate intake and secondarily on alcohol intake. Therefore, the conclusions of this study are based primarily on folate intake. Other dietary factors of importance for methyl donor intake (*e.g.*, vitamins B6 and B12) were not accounted for in this selection of patients.

Increasing the power of the study, including CRCs from patients with the complete spectrum of folate and alcohol intake and analysis of the effect of intake of other methyl donors such as vitamins B6 and B12, will reveal whether the observed effects of methyl donor deficiency is stable. In addition, it is necessary to study whether there is effect modification by alcohol (as suggested with overall CRC in the First National Health and Nutrition Examination Survey study; Ref. 7) in the complete group of CRCs derived from the NLCS. Another drawback of this study is that only case-case and not case-cohort analyses were performed. Knowledge on global hypomethylation status, which is hypothesized to occur previously to regional hypermethylation, would also be interesting to analyze with respect to promoter hypermethylation. In addition, it is possible that the observed effect of folate deficiency on promoter methylation will be stronger after stratification for functionally important SNPs in genes involved in folate metabolism. Methylenetetrahydrofolate reductase, methionine synthase, and methionine synthase reductase are interesting enzymes involved in the generation of S-adenosylmethionine, the primary methyl donor for DNA methylation reactions. All three enzymes have been reported to have common SNPs, which are associated with enhanced thermolability and decreased enzyme activity. The effect of the SNPs on colorectal and other cancer risks also seems to be dependent on methionine, folate, vitamins B6 and B12, and alcohol intake (34–40).

In conclusion, despite its limited size, this study suggests that methyl donor intake is associated with an increased frequency of promoter hypermethylation of genes involved in CRC carcinogenesis.

Table 4 Clinicopathological parameters and other patient characteristics in CRCs with no genes versus at least one gene methylated

	No genes methylated (n = 28)	At least one gene methylated (n = 94)	P
Clinicopathological parameters			
Dukes stage			
A	7/26 (27%)	23/89 (26%)	0.412
B	10/26 (39%)	29/89 (33%)	
C	4/26 (15%)	27/89 (30%)	
D	5/26 (19%)	10/89 (11%)	
Location of tumor			
Colon	19/28 (68%)	65/94 (69%)	0.675
Rectosigmoid	2/28 (7%)	11/94 (12%)	
Rectum	7/28 (25%)	18/94 (19%)	
Proximal location (yes)	19/28 (68%)	56/93 (60%)	0.465
Other patient characteristics			
Age at diagnosis (yr)	67.8	67.3	0.581
Gender (male)	11/28 (39%)	57/94 (61%)	0.046
Family history of cancer (yes)	7/28 (25%)	12/94 (13%)	0.117
Dietary factors			
Energy intake (kcal)	1937	1955	0.854
Fiber intake (g)	28.6	26.6	0.274
Vitamin C intake (mg)	115	104	0.339
Iron intake (mg)	13.2	12.6	0.368

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